Patent
Attorney's Docket No. 021565-060

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Patent Application of	
Peter Waterhouse et al.	Group Art Unit: 1635
Application No.: 09/287,632	Examiner: ZARA JANE
Filed: April 7, 1999	Confirmation No.: 6526
For: METHODS AND MEANS FOR OBTAINING MODIFIED PHENOTYPES	

Declaration of Dr. Marc De Block under 37 C.F.R. section 1.132

I, Marc De Block, hereby declare that:

1. I am a citizen and resident of Belgium. I received a Ph. D. degree in 1981 from the University of Ghent, Belgium.

2. Since 1984, I have been employed in Ghent, Belgium by PLANT GENETIC SYSTEMS N.V. or its successor firms AVENTIS CROPSCIENCE N.V. and BAYER BIOSCIENCE NV. My work has involved the supervision of projects studying plant growth and development as well as supervision of projects studying tissue specific gene-silencing.

3. I am familiar with the field of the plant molecular biology, particularly the fields of cell cycle research, plant development, apoptosis and stress resistance and I have authored and co-authored several scientific publications in these fields (a list of publications is submitted as ANNEX 1). I am also familiar with the field of post-transcriptional gene silencing and have applied such technology to achieve various traits as described in several scientific publications, e.g. those listed in ANNEX 1 as items 24 and 25.

4. I have read US Patent Application No. 09/287,632 (the "Application") including the presently pending claims.

5. I have been informed that Bayer BioScience N.V. has obtained a license to the inventions as described e.g. in the Application.

6. I have further been informed that in the Office Action dated 8 February 2007 issued by the United States Patent and Trademark Office in connection with the Application, the Examiner alleged that "The claim(s) contain subject matter which was not described in the specification in such a way as to reasonably convey to one skilled in the relevant art that the inventor(s), at the time the application was filed, had possession of the claimed invention."

7. It is my understanding that the Examiner believes that the specification, claims and the art do not adequately describe the distinguishing features or attributes shared by the members of the claimed genus of DNA constructs for gene silencing comprising the recited sense and antisense nucleotide sequences and further comprising an intron sequence, whereby any intronic sequence is inserted anywhere in the chimeric DNA, and whereby the DNA construct provides for the function claimed, namely reducing the phenotypic expression of a nucleic of interest in any plant cell or in any isolated eukaryotic cell.

8. The Examiner also asserts that the Application provides only two examples of chimeric constructs which contain an intron. Thus, the Examiner believes that the Application does not describe a sufficient number of different possibilities for the chimeric dsRNA constructs claimed to allow a person skilled in the art to conclude that the inventors were in possession of the genus as broadly claimed.

9. I disagree with the Examiner for the following reasons.

10. In the period of 1999-2003, I supervised and participated in a project at Bayer BioScience N.V., together with Dr Marina Byzova aiming at reducing the adsorption or reflection of photosynthetically active radiation by the bright yellow flowers of oilseed rape. We investigated whether hairpin RNA-mediated gene silencing could be implemented to silence B-type MADS-box floral organ identity genes in a tissue-specific manner (i.e. in the second whorl only) to transform the yellow petals into green sepaloid structures in Arabidopsis and oilseed rape. The results of this project are summarized in the peer-reviewed publication listed as item 24 in my publication list and attached to this declaration as ANNEX 2.

11. The design of the hairpin RNA encoding chimeric genes is described in detail on page 380 right column of ANNEX 2, in the section entitled "Plasmid construction". The hairpin RNA encoding chimeric genes contained either a 3' end of the *B. napus* homologue of APETALA3 ("AP3") or of the PISTILLATA ("PI") gene of A. thaliana, cloned in inverse orientation so that upon transcription a double stranded RNA molecule or hairpin RNA molecule could be formed. Both AP3 and PI are examples of B-type MADS-box floral organ identity genes.

12. At the time of designing the hairpin RNA encoding chimeric genes we were aware --due to our collaboration with CSIRO, assignee of the Application-- of the results indicated in the Application at least on page 23 and in Example 6 that the inclusion of an intron in the transcribed region enhances the efficiency of the reduction of expression of the gene of interest. Therefore, we decided to include an intron in at least one of the chimeric constructs (indicated in ANNEX 2 as pAP1::hpBPI) that were to be used in the project.

13. Since I did not perceive the identity of the particular intron to be used to be critical to achieve the same effect, another intron which was readily available at our premises was used i.e. intron IV2 from the potato light-inducible tissue-specific gene ST-LS1 as described by Vancanneyt et al., 1990, Mol. Gen. Genet. 220:

245-250 (see ANNEX 2, page 380, 8-9 lines from the bottom of the right-hand column).

14. Interestingly, the hairpin RNA encoding construct comprising the intron (pAP1::hpBPI) is the most efficient in Arabidopsis to achieve the desired double sepaloid phenotype (ANNEX 2, page 381 right hand column lines 26-27) and is the only construct when used in oilseed rape resulting in flowers with an apetalous or partially apetalous phenotype, while no differences in flowering were observed with the intron-less construct (ANNEX 2, paragraph spanning pages 382 and 383). Further modifications of the promoter driving the intron comprising hairpin RNA constructs finally resulted in the oilseed rape with sepaloid petals.

15. I believe the above described clearly illustrates that the invention described in the Application was in my mind not limited to the particular exemplified intron, as I immediately decided to exchange it for another known intron. I believe that a person of ordinary skill in the art reading the Application at the time the Application was filed would have reached a similar understanding.

16. In my view a person of ordinary skill in the art for the purpose of the inventions described in the Application is a person with a doctoral degree in the field of molecular biology of eukaryotes with at least some years of post doctoral research experience.

17. It is also my opinion that a person of ordinary skill in the art would have immediately understood that the applicability of the inventions described in the Application is not limited to the specific Example therein, nor to the particular intron used in that Example. The person of ordinary skill in the art would have understood that the exemplified intron could be exchanged for other well known introns, and that such other introns would function in the same manner, since the process of removal of introns from RNA molecules is a conserved process.

18. In conclusion, it is therefore my opinion that a person of ordinary skill in the art would have reasonably concluded, judged at the filing date, that the Application adequately described the claimed subject matter, to convey that the inventors were in possession of the invention as broadly claimed.

I also declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that wilful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such wilful false statements may jeopardise the validity of this application or any patent issued thereon.

06/08/07
Date

Marc De Block

ANNEX 1

LIST OF PUBLICATIONS

1. **Cornelissen, M.J., De Block, M., Van Montagu, M., Leemans, J., Schreier, P.H., Schell, J.** (1987) Plastid transformation: a progress report. In 'Plant DNA infectious agents'. Eds. Hohn, T., Schell, J.. Springer- Verlag/Wien. , 311-320

2. **D'Halluin, K., De Block, M., Denecke, J., Janssens, J., Leemans, J., Reynaerts, A., Botterman, J.** (1992) The *bar* gene as selectable and screenable marker in plant engineering. Methods in Enzymology. Ed. Ray Wu. Academic Press. *216*, 415-426

3. **De Block, M.** (1988) Genotype-independent leaf disc transformation of potato (Solanum tuberosum) using *Agrobacterium tumefaciens*. Theor. Appl. Genet. *76*, 767-774

4. **De Block, M.** (1990) Factors influencing the tissue culture and the *Agrobacterium tumefaciens*-mediated transformation of hybrid aspen and poplar clones. Plant Physiol. *93*, 1110-1116

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6. **De Block, M.** (1995) In situ enzyme histochemistry on plastic-embedded plant material. In Methods in Plant Cell Biology. Eds. Galbraith, D.W., Bohnert, H.J., Bourque, D.P.. Academic Press, London. *49(A)*, 153-163

7. **De Block, M., Botterman, J., Vandewiele, M., Dockx, J., Thoen, C., Gosselé, V., Movva, N.R., Thompson, C., Van Montagu, M., Leemans, J.** (1987) Engineering herbicide resistance in plants by expression of a detoxifying enzyme. EMBO J. *6*, 2513-2518

8. **De Block, M., De Sonville, A., Debrouwer, D.** (1995) The selection mechanism of phosphinothricin is influenced by the metabolic status of the tissue. Planta *197*, 619-626

9. **De Block, M., Debrouwer, D.** (1991) Two T-DNA's co-transformed into *Brassica napus* by a double Agrobacterium tumefaciens infection are mainly integrated at the same locus. Theor. Appl. Genet. *82*, 257-263

10. **De Block, M., Debrouwer, D.** (1992) In-situ enzyme histochemistry on plastic-embedded plant material. The development of an artefact-free ß-glucuronidase assay. Plant J.l *2*, 261-266

11. **De Block, M., Debrouwer, D.** (1993a) Engineered fertility control in transgenic Brassica napus L.: Histochemical analysis of anther development. Planta *189*, 218-

12. **De Block, M., Debrouwer, D.** (1993b) RNA-RNA in situ hybridization using digoxigenin-labeled probes: the use of high-molecular-weight polyvinyl alcohol in the alkaline phosphatase indoxyl-nitroblue tetrazolium reaction. Anal. Biochem. *215*, 86-89

13. **De Block, M., Debrouwer, D.** (1996) RNA-RNA in situ hybridization using DIG-labeled probes: the effect of high molecular weight polyvinyl alcohol on the alkaline phosphatase indoxyl-nitroblue tetrazolium reaction. In Nonradioactive in situ hybridization application manual. Boehringer Mannheim. , 141-145

14. **De Block, M., Debrouwer, D., Moens, T.** (1997) The development of a nuclear male sterility system in wheat. Expression of the *barnase* gene under the control of tapetum specific promoters. Theor. Appl. Genet. *95*, 125-131

15. **De Block, M., Debrouwer, D., Tenning, P.** (1989) Transformation of *Brassica napus* and *Brassica oleracea* using *Agrobacterium tumefaciens* and the expression of the *bar* and *neo* genes in the transgenic plants. Plant Physiol. *91*, 694-701

16. **De Block, M., Herrera-Estrella, L., Van Montagu, M., Schell, J., Zambryski, P.** (1984) Expression of foreign genes in regenerated plants and their progeny. EMBO J. *3*, 1681-1689

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18. **De Greef, W., Delon, R., De Block, M., Leemans, J., Botterman, J.** (1989) Evaluation of herbicide resistance in transgenic crops under field conditions. Bio/Technology *7*, 61-64

19. **Haring, M.A., De Block, M.** (1990) New roads towards chloroplast transformation in higher plants. Physiol. Plant. *79*, 218-220

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22. **Reynaerts, A., De Block, M., Hernalsteens, J.-P., Van Montagu, M.** (1988) Selectable and screenable markers. Plant Molecular Biology Manual. Kluwer

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24. **Byzova, M., Verduyn C, De Brouwer D, De Block, M.** (2004) Transforming petals into sepaloid organs in Arabidopsis and oilseed rape: implementation of the hairpin RNA –mediated gene silencing technology in an organ-specific manner. Planta 218: 379-387.

25. **De Block M, Verduyn C, De Brouwer D, Cornelissen M** (2005) Poly(ADP-ribose) polymerase in plants affects energy homeostasis, cell death and stress tolerance; Plant J. 41(1):95-106

26. **Herrera-Estrella, L., De Block, M., Zambryski, P., Van Montagu, M., Schell, J.** (1985) Agrobacterium as a vector system for the introduction of genes into plants. In: Dodds, J.H. (Ed.) Plant Genetic Engineering. Cambridge University Press, 61-93.

27. **De Block, M., De Brouwer, D.** (2002) A simple and robust in vitro assay to quantify the vigour of oilseed rape lines and hybrids. Plant Physiol. Biochem. 40: 845-852.

28. **Nelissen, H., Clarke, J.H., De Block, M., De Block, S., Vanderhaeghen, R., Zielinski, R.E., Dyer, T., Lust, S., Inzé, D., Van Lijsebettens, M.** (2003) DRL1, a homolog of the yeast TOT4/KT112 protein, has a function in meristem activity and organ growth in plants. Plant Cell 15: 639-654.

29. **De Block M, Verduyn C, De Brouwer D, Cornelissen M** (2004) Generating stress tolerant crops by economizing energy consumption. Pflanzenschutz-Nachrichten Bayer 57(1): 105-110.

Planta (2004) 218: 379–387
DOI 10.1007/s00425-003-1117-1

ANNEX 2

ORIGINAL ARTICLE

Marina Byzova · Christoph Verduyn · Dirk De Brouwer
Marc De Block

Transforming petals into sepaloid organs in *Arabidopsis* and oilseed rape: implementation of the hairpin RNA-mediated gene silencing technology in an organ-specific manner

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Abstract Oilseed rape (*Brassica napus* L.) genotypes with no or small petals are thought to have advantages in photosynthetic activity. The flowers of field-grown oilseed rape form a bright-yellow canopy that reflects and absorbs nearly 60% of the photosynthetically active radiation (PAR), causing a severe yield penalty. Reducing the size of the petals and/or removing the reflecting colour will improve the transmission of PAR to the leaves and is expected to increase the crop productivity. In this study the 'hairpin' RNA-mediated (hpRNA) gene silencing technology was implemented in *Arabidopsis thaliana* (L.) Heynh. and *B. napus* to silence B-type MADS-box floral organ identity genes in a second-whorl-specific manner. In *Arabidopsis*, silencing of B-type MADS-box genes was obtained by expressing *B. napus APETALA3* (*BAP3*) or *PISTILLATA* (*BPI*) homologous self-complementary hpRNA constructs under control of the *Arabidopsis* A-type MADS-box gene *APETALA1* (*API*) promoter. In *B. napus*, silencing of the *BPI* gene family was achieved by expressing a similar hpRNA construct as used in *Arabidopsis* under the control of a chimeric promoter consisting of a modified petal-specific *Arabidopsis AP3* promoter fragment fused to the *API* promoter. In this way, transgenic plants were generated producing male fertile flowers in which the petals were converted into sepals (*Arabidopsis*) or into sepaloid petals (*B. napus*). These novel flower phenotypes were stable and heritable in both species.

Keywords Apetalous · *Arabidopsis* · *Brassica* · Double sepaloid · MADS-box · Petal

M. Byzova · C. Verduyn · D. De Brouwer
M. De Block (✉)
Bayer BioScience N.V., Jozef Plateaustraat 22,
9000 Gent, Belgium
E-mail: marc.deblock@bayercropscience.com
Fax: + 32-9-2240694

Abbreviations *PAR*: photosynthetically active radiation · *ST-LS1*: potato light-inducible tissue-specific ST-LS1 gene · *GUS*: β-glucuronidase

Introduction

Flowers of oilseed rape (*Brassica napus*) have four well-developed bright-yellow petals. During flowering time, flowers form a very bright-yellow layer that reflects and absorbs solar radiation. As consequence, only 24% of the photosynthetically active radiation (PAR) reaches the leaf canopy (Chapman et al. 1984). This accelerates leaf and bract senescence, reduces dry matter accumulation, and lowers seed set (Daniels et al. 1986).

A few strategies to improve the photosynthetic efficiency of oilseed rape by utilising different apetalous variants (Buzza 1983; Jiang and Becker 2003) or the *stamenoid petal* (stap) variant with flowers bearing staminoid petals (Fray et al. 1997) have been proposed. Physiological analyses have revealed the potential benefit of such a petalless flower phenotype on *B. napus* yield (Rao et al. 1991; Fray et al. 1995).

The currently used apetalous genotypes are controlled either by two recessive genes (Fray et al. 1996) or by an interaction of cytoplasmic genes and two pairs of nuclear genes (Jiang and Becker 2003). This genetic complexity makes it difficult to fully implement the apetalous trait into commercial rapeseed varieties. Additionally, the apetalous character appears to be unstable under field conditions at high temperatures and in long days (Rao et al. 1991). The *B. napus stap* variant also possesses poor agronomic attributes, such as deformed leaves and poor vigour (Fray et al. 1997).

A more promising strategy to improve PAR transmission in oilseed rape would be the use of a single dominant gene that converts the bright-yellow petals into small non-light reflecting structures such as sepals. Such an organ conversion is preferable over the removal of the petals to avoid interfering with insect pollination. Pierre et al. (1996) have shown that honeybees, the main

pollinators on oilseed rape, do not crawl over the anthers and stigma of apetalous flowers as they do in petalous ones but often insert their tongues between the sepals to collect the nectar. In this way pollination might be reduced, resulting in a lower seed set.

The molecular mechanisms governing floral organ identity are well understood. According to the "A–E" model, the organ identity of each floral whorl is determined by a unique combination of four organ identity activities, called A, B, C and E (Weigel and Meyerowitz 1994; Pelaz et al. 2000; Jack 2001; Theißen 2001; Fig. 1). Expression of the (A)-type genes specifies sepal formation. The combination of (A + B + E) activities specifies the formation of petals, while combined (B + C + E) functions specify stamen formation. Expression of the (C + E)-type genes determines the development of carpels (Fig. 1). All types of organ identity genes have been cloned from *Arabidopsis*. An example of the A-type gene is *API* (Mandel et al. 1992). The B-type genes are *AP3* (Jack et al. 1992) and *PI* (Goto and Meyerowitz 1994), and the C-type gene is *AGAMOUS* (*AG*) (Yanofsky et al. 1990). The E-function is provided by three *SEPALLATA* genes (Pelaz et al. 2000). All these genes are transcription factors belonging to the MADS-box gene family.

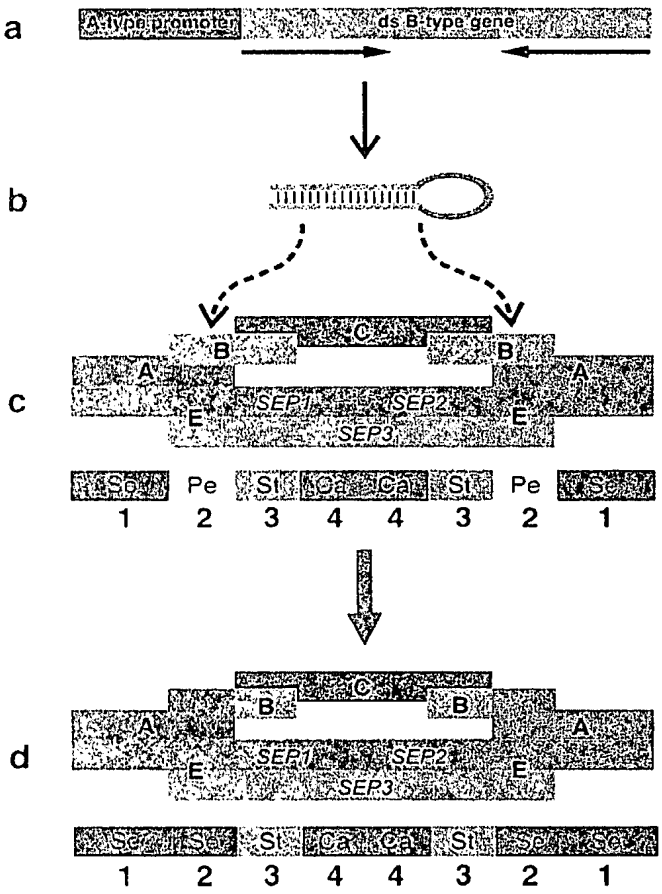

Fig. 1a–d Experimental strategy. a Representation of the basic construct used in this study. A DNA fragment of a 3′-coding region of a B-type MADS-box gene (*yellow box*) was sub-cloned as an inverted repeat (directions are indicated by *arrows*) with a part of the GUS gene or the intron IV2 from gene ST-LS1 (Vancanneyt et al. 1990) as a spacer (*blue box*). The constructs were driven by an A-type MADS-box gene promoter (*green box*). b Transcripts produced by the construct are predicted to form a hairpin structure. c Domains of the A, B, C and E functions and the corresponding organ identities in floral whorls in wild-type plants. d Domains of the A, B, C and E functions and the corresponding organ identities in floral whorls in transgenic plants. In transgenic plants, down-regulation of the B-type function in the second whorl only leads to development of sepals instead of petals. Numbers indicate whorls. *Se* sepals, *Pe* petals, *St* stamens, *Ca* carpels

In this paper, silencing of the B-type MADS-box genes in a second-whorl-specific manner was obtained in both *Arabidopsis* and *B. napus* flowers by expressing a *B. napus* B-type gene hpRNA construct under control of an *Arabidopsis* A-type MADS-box gene promoter (Fig. 1). In this way, *Arabidopsis* lines with double sepaloid flowers and *B. napus* lines with flowers in which petals are converted into sepaloid petals were generated. The novel flower phenotypes were stable and heritable in both species.

Materials and methods

Plant material

Arabidopsis thaliana (L.) Heynh. ecotype C24, kindly provided by Dr. M. Van Lijsebettens (VIB, Gent, Belgium), and the double haploid *Brassica napus* L. line cv. Simon (Bayer BioScience N.V., Gent, Belgium) were used in this study.

Plasmid construction

The 3′- coding regions of the *BAP3* and *BPI* genes were cloned by means of RT–PCR performed on total RNA isolated from *B. napus* flower buds. RT–PCR was performed according to the protocol of the SuperScript First-Strand Synthesis System for RT–PCR (Invitrogen). *AP3* cDNA-specific primers:

- 5′-CGCACTCAGATTAAGCAGAGGC-3′ and
- 5′-GGAAGGTAATGATGTCAGAGGC-3′

and *PI* cDNA-specific primers:

- 5′-GGGAGAAGATATACAGTCTCTCAAC-3′ and
- 5′-GAATCGGTTGCACTCTATATCC-3′

were chosen based on the published sequences (Jack et al. 1992, GenBank Accession D30807; Goto and Meyerowitz 1994, GenBank Accession M86337). In the pAP1::hpBAP3 construct, one of the *BAP3*-specific DNA fragments, 380-bp in length, was cloned as a inverted repeat with the β-glucuronidase (GUS) fragment containing nucleotides 744–975 as a spacer. In the pAP1::hpBPI construct, one of the *BPI*-specific fragments, 255-bp in length, was cloned as an inverted repeat with the intron IV2 from the potato light-inducible tissue-specific gene ST-LS1, 251-bp in length, as a spacer (Vancanneyt et al. 1990). In the pAP1::hpBAP3 and pAP1::hpBPI constructs, gene-specific structures were driven by a 1,182-bp fragment of the *API* promoter. The fragment of the *API* promoter (−1182 to +1) was cloned by means of PCR from pKY65 plasmid kindly provided by Martin Yanofsky. In pΔAP3-AP1::hpBPI fragments of the *AP3* promoter, containing nucleotides −727 to −556 and −224 to −1 were cloned by PCR based on

the published sequence (Irish and Yamamoto 1995, GenBank Accession U30729) and linked to the 5'-end of the *AP1* promoter. Plasmid constructs were introduced into *Agrobacterium tumefaciens* strain C58C1rif by electroporation.

Plant transformation

The transformations of *A. thaliana* and *B. napus* were essentially done as described by Valvekens et al. (1992) and De Block et al. (1989), respectively.

Cytology

The embedding was done in Historesin as advised by the manufacturer (Leica, Heidelberg, Germany). Sections 5 μm thick were stained with 0.05% toluidine blue.

In situ hybridization

Embedding in methacrylate, sectioning, and the removal of the plastic were essentially done as described by Baskin et al. (1992). The in situ hybridizations on 7-μm sections were essentially done as described by De Block and De Brouwer (1993).

Microscopy

Sections were examined with an Axioplan (Zeiss, Jena, Germany) microscope equipped with Normaski differential interference contrast.

Spectrophotometric determination of chlorophyll

The total chlorophyll (a + b) content was measured as described by Bruisma (1963).

Results

General strategy: silencing the B-type MADS-box genes in a second floral whorl-specific manner

To convert petals into sepals without interfering with anther development, the strategy outlined in Fig. 1 was used. Following the A–E flower development model it is expected that silencing of a B-type MADS-box gene, *AP3* or *PI*, in the second whorl will redirect the development of petals into sepals. This could be obtained by expressing in the second, but not in the third whorl self-complementary 'hairpin' RNA (hpRNA) constructs containing *AP3*- and/or *PI*-specific sequences. Down-regulation of the B-type MADS-box genes in the third whorl has to be avoided to maintain normal male fertility. For this purpose an A-type promoter driving the expression of the hpRNA construct could be used.

Starting from the *PI* and *AP3* sequences (Jack et al. 1992; Goto and Meyerowitz 1994), we identified in the amphidiploid *B. napus* five *AP3*-like (*BAP3*) and three *PI*-like (*BPI*) genes that were actively expressed during flower development (data not shown). Fragments of the 3'-coding region of the *BAP3* and *BPI* genes were isolated. The nucleotide sequence similarity between members of the same B-type MADS-box gene subfamily turned out to be on average 95%. Each *B. napus* gene subfamily shared with its unique *Arabidopsis* counterpart about 91% sequence similarity, containing multiple blocks of more than 20 bases of perfect homology. This high sequence similarity should be sufficient to silence the target genes in both *Arabidopsis* and *B. napus* by using the same hpRNA constructs (Helliwell and Waterhouse 2003). The feasibility of the strategy to convert petals into sepals by silencing the B-type MADS-box genes only in the second floral whorl was first evaluated in the model plant *Arabidopsis thaliana*.

Generation of *Arabidopsis* transgenic lines with male fertile double sepaloid flowers

To make constructs that produce hpRNA B-type MADS-box gene transcript, the 3'-coding regions of one *BAP3* and one *BPI* gene, were subcloned as an inverted repeat (see Materials and methods). Both hpBAP3 and hpBPI gene-specific sequences were driven by a 1.1-kb promoter fragment of the *Arabidopsis AP1* gene. The resulting pAP1::hpBAP3 and pAP1::hpBPI constructs were introduced separately into *Arabidopsis*.

A total of 125 pAP1::hpBAP3 and 56 pAP1::hpBPI transgenic lines was generated. All the plants were normal in terms of vegetative growth while they had morphological changes in flower organs. 16.9% of the pAP1::hpBPI and 5.6% of the pAP1::hpBAP3 lines exhibited the desirable double sepaloid phenotype (Fig. 2b). Instead of petals, sepals developed in the second floral whorl, indistinguishable from those of the first whorl except for their slightly smaller size. Despite their transformation, these organs developed in the positions and on a time course characteristics of petals. Some other pAP1::hpBAP3 T_0 plants had a range of phenotypes related to the severity of homeotic transformations observed in petal and stamen development. 10.4% of the pAP1::hpBAP3 lines produced flowers with short white petals and 20% of the lines had homeotic aberrations in stamens ranging from weak carpelloidy to complete transformation of stamens into carpels (Table 1). In contrast to the pAP1::hpBAP3 lines, no aberrations in the third floral whorl were observed in the pAP1::hpBPI transgenic plants (Table 1).

Microscopic analysis of cross-sections of mature pAP1::hpBPI double sepaloid flowers revealed that the mesophyll cells of the second-whorl organs were sepaloid in nature, as indicated by the presence of chloroplasts and their larger size than those normally found in wild-type petals. The abaxial epidermis was like that of sepals, consisting of stomata and irregularly shaped cells (Fig. 2d). The same results were obtained for pAP1::hpBAP3 double sepaloid flowers (data not shown).

To confirm that the double sepaloid phenotype of *Arabidopsis* transgenic plants was caused by depletion of expression of endogenous B-type homeotic genes in the

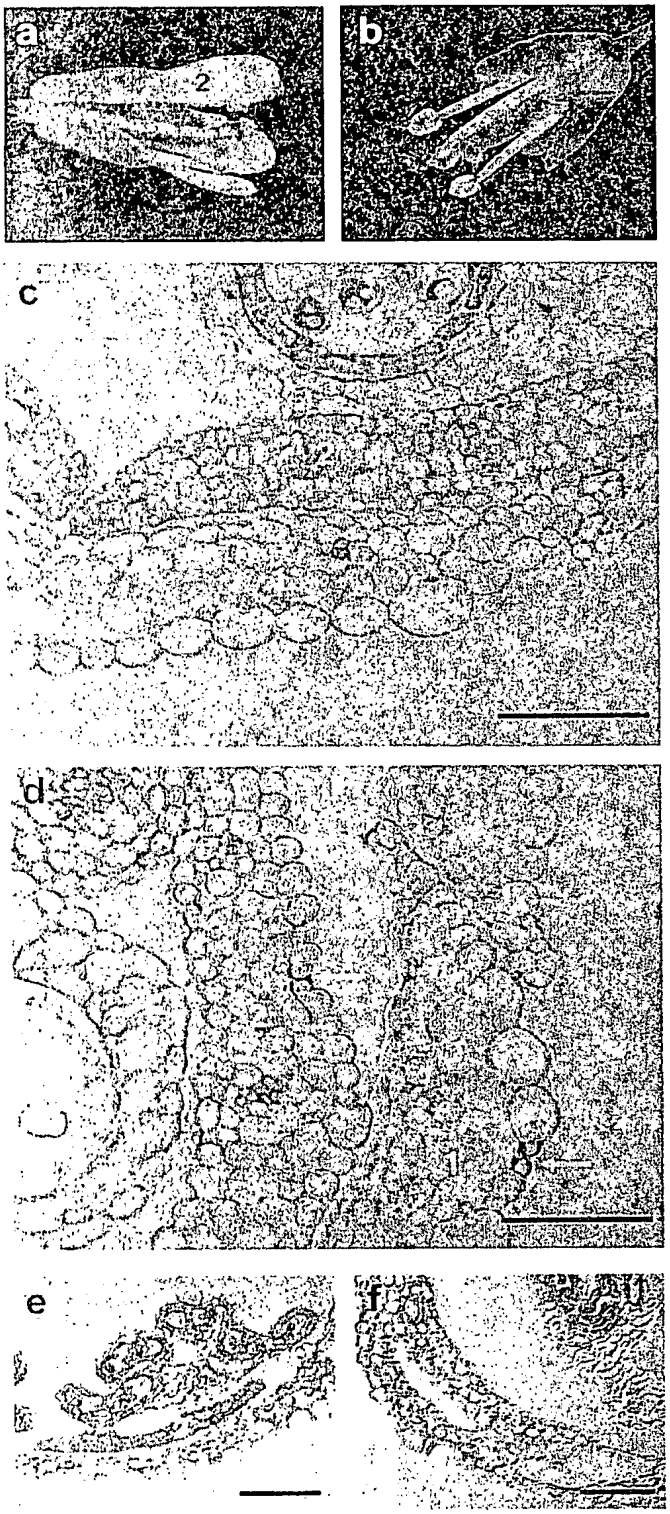

Fig. 2a–f Analysis of the double sepaloid pAP1::hpBPI *Arabidopsis thaliana* flowers. **a** C24 *Arabidopsis* wild-type mature flower. **b** Mature transgenic flower. Second-whorl organs are sepals (*arrow*) that are slightly smaller than the true sepals. **c, d** Cytological transverse sections taken approximately in the middle of anthers of flower buds at stage 12. **c** Cellular morphology of first- and second-whorl organs of a wild-type flower. Mesophyll cells of the second-whorl petals are smaller than those of sepals developed in the first whorl. Abaxial epidermal cells of petals are regular in shape. **d** Cellular morphology of first- and second-whorl organs of a transgenic flower. Mesophyll and epidermal cells of the second-whorl organs are slightly smaller in size than cells of the first-whorl sepals. The shape of the cells of the second-whorl sepals is similar to those of the first-whorl sepals. Stomata (*arrows*) are present in the abaxial epidermis of the second-whorl organs as in normal first-whorl sepals. **e, f** In situ analysis of *PI* expression in transverse sections of wild-type and transgenic flowers. The hybridisation signal is confined to the second- and the third-whorl organs in wild-type flowers (**e**). In transgenic flowers (**f**) *PI* expression is detected in the third-whorl organs only. Numbers indicate whorls. Bars = 50 μm (**c, d, f**), 100 μm (**e**)

second whorl, the *PI* mRNA expression pattern in pAP1::hpBPI was examined by in situ hybridization. In wild-type *Arabidopsis* flowers, *PI* mRNA is detected from stage 3 (Smyth et al. 1990) in second- third- and fourth-whorl primordia. In the second and third whorls it persists until anthesis (Goto and Meyerowitz 1994). In pAP1::hpBPI double sepaloid flowers the *PI* transcript was not detectable in the second-whorl primordia at any of the stages examined (Fig. 2f). Expression of *PI* in developing stamens was similar to that observed in the wild type.

As it has been shown that expression of both *PI* and *AP3* genes is reduced when either the *PI* or *AP3* gene is mutated (Jack et al. 1992; Goto and Meyerowitz 1994), we anticipated that inhibition of expression of one of the B-type MADS-box genes in a tissue where both genes are active would lead to reduction of expression of the counterpart gene in the same manner. To verify this hypothesis, in situ hybridization of the pAP1::hpBPI flowers using the *BAP3*-specific fragment as a probe was performed. As was predicted, *AP3* RNA was not detected in the second whorl of developing organs. However, no reduction in the level of the *AP3* mRNA was observed in stamens (data not shown).

Heritability and stability of the double sepaloid trait was tested by self-pollination. The trait was heritable and in the case of pAP1::hpBPI stable through the T_1 and T_2 generations. In the case of pAP1::hpBAP3 some T_1 and T_2 lines produced flowers with homeotic aberrations in stamens, as previously observed in the T_0 plants.

In *B. napus*, silencing of B-type MADS-box genes in the second whorl results in the transformation of petals into sepaloid petals

To evaluate whether the expression of the pAP1::hpBAP3 and pAP1::hpBPI genes would also result in a double sepaloid phenotype in *B. napus*, 48 and 53 transgenic lines, respectively, were generated.

All the pAP1::hpBAP3 lines had wild-type flowers. Among the pAP1::hpBPI transgenic lines, 22.6%

Table 1 Phenotypic analysis of T_0 *Arabidopsis thaliana* plants

[a]Range of aberrations in stamens from mild to complete conversion of stamens into carpels

Transformed constructs	Total number of transgenic lines	Plants with mutant phenotype (%)		
		Double sepaloid Fertile	Double sepaloid Partially male sterile[a]	Short petals Fertile
pAP1::hpBPI	56	16.9	<2	18.9
pAP1::hpBAP3	125	5.6	20	10.4

exhibited an apetalous or partially apetalous phenotype characterised by the appearance of flowers without petals or bearing 1, 2 or 3 petals only (Table 2). Frequently, the petals were significantly smaller and narrower than those from wild type (data not shown). However, this phenotype was unstable and not heritable.

The absence of the double sepaloid phenotype in transgenic *B. napus* lines with the same constructs used in *Arabidopsis* could be due to an inability of the *Arabidopsis AP1* promoter to direct transcription of adequate amounts of double-stranded transcripts necessary to trigger silencing of all target *BAP3* or *BPI* genes expressed in rapeseed flowers. Starting from this hypothesis, a new construct was generated that could produce higher amounts of hpRNA. Because the pAP1::hpBAP3 *B. napus* transgenic plants did not exhibit any phenotypes different from those of wild-type plants, and in *Arabidopsis* the most stable double sepaloid flower phenotype was obtained with the hpBPI construct, we continued only with the hpRNA *BPI* gene.

To enhance the level of expression of hpBPI specifically in the second whorl, an *Arabidopsis* modified *AP3* regulatory fragment was added to the *AP1* promoter.

Discrete *cis*-acting elements regulating spatial and temporal expression of the *Arabidopsis AP3* gene have been identified (Hill et al. 1998; Tilly et al. 1998). Based on these data the positive regulator of the *AP3* expression during the early stages of flower development was combined with the petal-specific regulatory region (see Materials and methods). The modified *AP3* promoter was introduced in the pAP1::hpBPI construct directly upstream of the *AP1* sequence. This pΔAP3-AP1::hpBPI construct was transformed into *B. napus*.

Of the 125 primary transformants, 11.2% produced flowers with aberrant second-whorl organs. Of these 11.2% lines, half (5.6%) produced flowers in which petals were converted into sepaloid petals (Fig. 3a, Table 2). These organs were yellowish-green, indicating the presence of chloroplasts in their cells that is characteristic of wild-type sepals. The size of the sepaloid petals was comparable to the size of true sepals. These organs were narrow and almost strap-like in shape, like sepals, but had a small lamina and base, characteristic of a petal. In addition the lamina portion was wrinkled (Fig. 3a).

The aberrant *B. napus* flowers with sepaloid petals were analysed microscopically to verify the identity of tissues in the second-whorl organs. As shown in Fig. 3c the size and the shape of epidermal and mesophyll cells of these organs were indistinguishable from the first-whorl sepals. Moreover, the mesophyll cells of the sepaloid petals contained a large number of chloroplasts (Fig. 3d).

In addition, spectrophotometric analysis of chlorophyll fluorescence, which was done on the first and the second floral organs of transgenic plants, revealed that chlorophyll content in the sepaloid petals is only 30% less than in the true wild-type sepals (data not shown).

In situ hybridization of flower sections with a *BPI*-specific probe confirmed the absence of a detectable level of *BPI* gene expression in the second whorl of the transgenic flowers, indicating that the complete *BPI* gene family was down-regulated (Fig. 3e).

The other half of the 11.2% transgenic pΔAP3-AP1::hpBPI lines exhibited partial apetalous and apetalous phenotypes similar to those observed in pAP1::hpBPI transgenic plants (Table 2).

The flower phenotype with sepaloid petals is a stable trait in *B. napus* transgenic plants

The stability of transformation of petals to sepaloid petals in *B. napus* was tested for six lines, of which the original T_0 plants had flowers with sepaloid petals and contained only one copy the pΔAP3-AP1::hpBPI transgene. The T_0 plants were first maintained by selfing. The transgenic plants of these T_1 generations had flowers with sepaloid petals while the azygous segregants had normal wild-type flowers. For each line ten transgenic plants of the T_1 generation were backcrossed with the original non-transgenic double haploid *B. napus* line cv.

Table 2 Phenotypic analysis of T_0 *Brasssica napus* plants

Transformed constructs	Total number of transgenic lines	Plants with mutant phenotype (%)	
		Sepaloid petals	Apetalous/partially apetalous
pAP1::hpBAP3	48	0	0
pAP1::hpBPI	53	0	22.6
pΔAP3-AP1::hpBPI	125	5.6	5.6

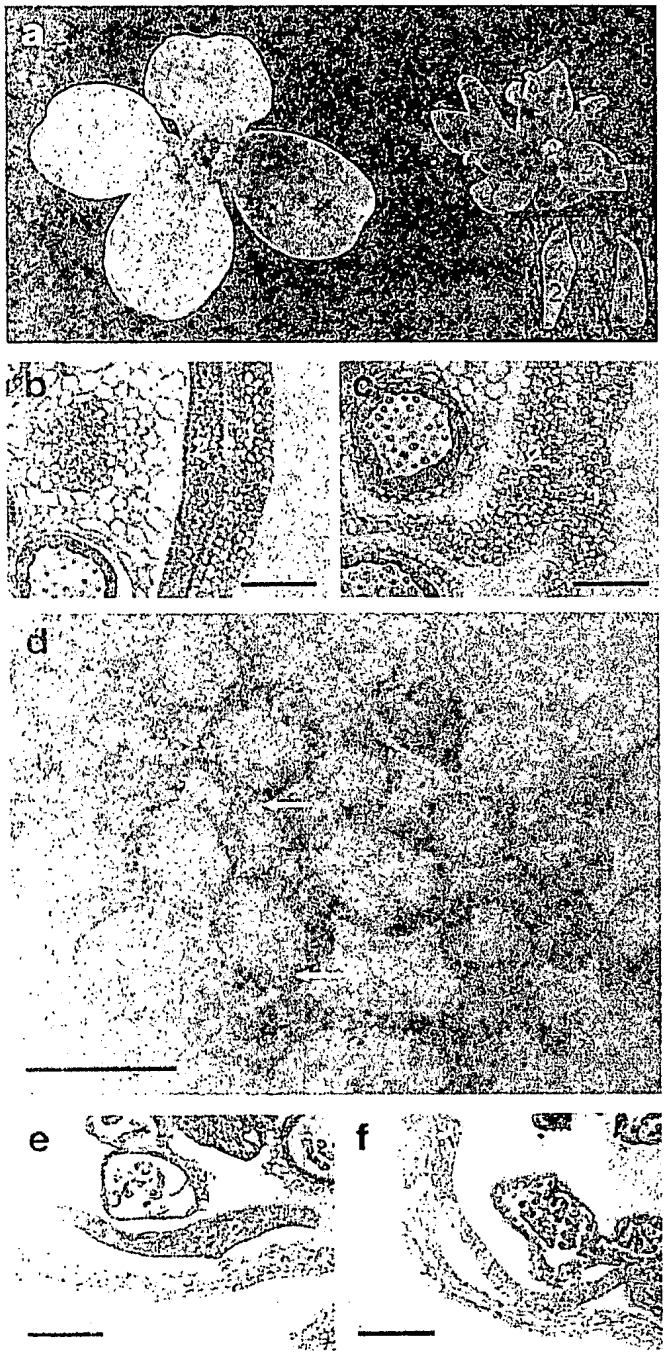

Fig. 3a–e Analysis of the pΔAP3-AP1::hpBPI *B. napus* flowers. **a** Morphological features of *Brassica napus* flowers: mature wild-type flower (*left*), mature flower of a transgenic plant (*right*). The second-whorl organs of a transgenic flower are yellowish-green sepaloid petals (*arrow*). The size of these organs is similar to sepals developed in the first whorl, but the lamina-base structure can still be distinguished (for comparison see the detached organs in the *bottom right corner*: the second-whorl organ (*left*), the first-whorl organ (*right*) of a transgenic flower). **b–d** Cytological transverse sections taken approximately in the middle of anthers at the early yellow bud stage (Smith and Scarisbrick 1990). **b** Cellular morphology of first- and second-whorl organs of a wild-type flower. Mesophyll cells of petals are smaller than those of sepals. Epidermal cells of petals are regular in shape. **c** Cellular morphology of the first- and second-whorl organs of a transgenic plant. The shape and the size of mesophyll and epidermal cells of the second-whorl organs are similar to those of the first-whorl sepals. **d** Cytology of a sepaloid petal showing the presence of chloroplasts (two examples indicated by *arrows*) in the mesophyll cells. **e, f** In situ analysis of *BPI* expression on transverse sections of wild-type and transgenic flowers. The hybridization signal is confined to the second- and the third-whorl organs in wild-type flowers (**e**). In transgenic flowers (**f**) *BPI* expression is detected in the third-whorl organs only. Numbers indicate whorls. Bars = 20 μm (**d**), 100 μm (**b, c**), 200 μm (**e, f**)

Simon. Depending on whether the T_1 plant used was homo- or heterozygous for the transgene, all or 50% of the F_1 plants, respectively, had sepaloid petals in their flowers. A second backcross was done with 15 plants of each line. As expected, in the F_2 generations there was a 1:1 segregation of wild-type plants and plants with sepaloid petals. The flower phenotype of the transgenic F_2 plants was identical to those of the T_0, T_1 and F_1 transgenic plants.

Discussion

The hpRNA-mediated gene silencing technology has been proven to be a very efficient tool for gene discovery and functional genomics in diverse organisms such as fungi (Pickford et al. 2002), nematodes (Bargmann 2001), and animals (Harborth et al. 2001). In plants this technology has been used successfully to generate virus resistance (Waterhouse et al. 1998) as well as to obtain consistent and profound inhibition of the expression of transgenes and endogenous genes (Levin et al. 2000; Smith at al. 2000; Wesley et al. 2001; Liu et al. 2002).

Chuang and Meyerowitz (2000) demonstrated that the hpRNA-mediated silencing technology could be used to interfere with flower development. A range of aberrant flower phenotypes was obtained by down-regulating the floral organ genes *AGAMOUS*, *CLAVATA3*, *APETALA1*, and *PERIANTHIA* using hpRNA constructs driven by the constitutive 35S and nopaline synthase promoters. Recently, it has been shown that the hpRNA-mediated silencing technique can be used to silence genes in an organ-specific way. The fatty acid composition of *Arabidopsis* and cotton seeds was modified by down-regulating the seed expression of two fatty acid desaturase genes using hpRNA constructs driven by seed-specific promoters (Liu et al. 2002; Stoutjesdijk et al. 2002).

In this article we present for the first time to our knowledge implementation of the hpRNA-mediated technology to silence a multigene family in a floral whorl-specific manner. Silencing of the B-type MADS-box genes that are present in single copy in *Arabidopsis* but are present in multiple copies in *B. napus* causes complete transformation of petals to sepals in *Arabidopsis* and partial transformation in *B. napus*. This flower phenotype is stable and heritable in both species.

In *Arabidopsis*, unlike the silencing of the *PI* gene, silencing of the *AP3* gene results in homeotic aberrations in anthers in 20% of the cases. This implies that in these lines partial silencing of the *AP3* gene also occurs in developing stamens. These results can be attributed to two possibilities. First, in wild-type *Arabidopsis* flowers *API* is expressed during early floral stages throughout all four whorls and is down-regulated in whorls 3 and 4 by the *AG* gene during stage 3, persisting in whorls 1 and 2 only (Mandel et al. 1992; Bowman at al. 1993). However, in contrast to the endogenous promoter, the smaller *API* promoter fragment we used might have some activity in the central whorls after stage 3 as proposed by Yun et al. (2002). The activity of the pAP1::hpBAP3 gene might have led to down-regulation of *AP3* in the third floral whorl. Alternatively, an aberrant stamen development in the pAP1::hpBAP3 transgenic plants might be the result of the spreading of a silencing signal between floral whorls.

Both hypotheses imply that a certain amount of dsRNA of the *AP3* gene present in the third whorl of transgenic flowers is sufficient to trigger silencing of *AP3*. This is not the case for the *PI* gene, for which the down-regulation did not result in aberrant anther phenotype. *PI* and *AP3* are both expressed in developing petals and stamens. However *PI* expression levels are similar in both whorls, whereas *AP3* expression is lower in developing stamens than in petals (Zhou et al. 2002). It may be that for this reason a lower threshold concentration of hpRNA is required in stamens to provoke a partial inhibition of the *AP3* gene expression.

Although systemic spreading of silencing may be a concern for implementation of the hpRNA-mediated silencing technology in tissue-specific applications in plants (Wang and Waterhouse 2002), the stability of the aberrant flower phenotype throughout development of our transgenic plants indicates that at least in the case of the B-type MADS-box genes there is no significant spreading of silencing between the meristems of adjacent floral organs.

Another phenomenon that might limit application of the hpRNA gene silencing technique is spreading of RNA targeting. During this process spreading of the RNA silencing signal occurs from the initial target sequence into the adjacent 5′ and 3′ regions (Jones at al. 1999; Vaistij at al. 2002). This may result in the participation of the entire transcribed region of the target gene in the RNA silencing process. As a consequence, expression of other homologous genes can be inhibited. Based on this hypothesis and the fact that different types of MADS-box genes share a high percentage of homology at the MADS-box regions (Purugganan et al. 1995), target-site spreading along the *AP3* or *PI* transcribed sequences would led to silencing of not only *AP3* and *PI* but also of other MADS-box genes that are expressed in the developing second-whorl organs. In this case petals will be converted not only into sepals but also into organs with staminoid and/or carpeloid and/or other aberrant structures. The absence of such phenotypes in our transgenic plants suggests that silencing of B-type MADS-box genes was not associated with the spreading of RNA targeting. The absence of the target-site spreading process was also observed by Vaistij et al. (2002) for the ribulose-1,5-bisphosphate carboxylase/oxygenase and phytoene desaturase genes. These results demonstrate that the hpRNA-mediated gene silencing technology can be applied not only to silence all genes of a multigene family but also to silence specifically a single member of a subfamily or even of a multigene family.

B. napus plants transformed with the improved pΔAP3-AP1::hpBPI construct have small yellowish-green sepaloid petals in the second whorl. Although mesophyll and epidermal cells of these sepaloid petals are sepaloid in morphology, the light-yellow colour suggests that some petal-specific biochemical pathways are still active in the cells of these organs. In addition, the small lamina and base of these organs are petal characteristics. It might be that undetectable levels of *BPI* transcripts are still sufficient for maintenance of some petaloid features.

Recently, in *Arabidopsis* an alternative approach was used to interfere with the expression of *AP3* in a second-whorl-specific manner (Guan et al. 2002). A zinc finger protein designed to bind to a region upstream of *AP3* was fused to the human transcriptional repression domain of mSIN3. When the *API* promoter was used to drive the expression of this artificial zinc finger transcription factor, flowers were obtained that were partially apetalous or that contained some sepaloid petals. Although the use of synthetic transcription factors is a promising approach to interfere with gene regulation, high expression levels of these transcription factors are probably needed to obtain a full phenotype by gene repression. Due to technical limitations the use of such artificial transcription factors is less feasible when multiple genes with redundant function, like the B-type MADS-box genes in *B. napus*, have to be repressed.

Theoretically, in *Arabidopsis* a double sepaloid flower phenotype may also be obtained by silencing the *SEPALLATA* genes in the second whorl (Fig. 1). However, due to the redundant function of the *SEPALLATA* genes, all three genes would have to be silenced together (Pelaz et al. 2000).

In conclusion, *Arabidopsis* and *B. napus* lines with a flower phenotype that is, respectively, double sepaloid or has sepaloid petals, and that is male fertile and stable in subsequent generations can be obtained by a hpRNA-mediated gene silencing of the *PISTILLATA* gene exclusively in the second floral whorl. Further physiological studies of *B. napus* transgenic lines will allow quantification of the effect of the flower architecture with sepaloid petals on the distribution of PAR and on other important agronomic features such as pollination and overall seed yield.

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